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Susceptibility of Filamentous Fungi to Voriconazole Tested by Two Microdilution Methods

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The growing number of fungal infections, coupled with emerging resistance to classical antifungal agents, has led to the development of new agents, among them voriconazole. Susceptibility to voriconazole was tested by two microdilution techniques: the National Committee for Clinical Laboratory Standards reference method M38-A and a colorimetric method, Sensititre YeastOne. The study tested a total of 244 isolates: 223 *Aspergillus* (136 *Aspergillus fumigatus*, 37 *A. niger*, 26 *A. terreus*, 16 *A. flavus*, 7 *A. nidulans*, and 1 *A. ustus*), 14 *Fusarium* (8 *Fusarium moniliformis*, and 6 *F. oxysporum*), 6 *Scedosporium apiospermum*, and 1 *Rhizomucor pusillus* strain and four control strains (*Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *A. fumigatus* ATCC 204305, and *A. flavus* ATCC 204304). For all tested species except one *F. moniliforme* strain and *R. pusillus*, the MIC, the MIC at which 50% of the isolates are inhibited (MIC_{50}), and MIC_{90} ranges of ≤1 μg/ml were obtained for voriconazole, indicating excellent activity against these species. The high rate of agreement between the two methods used (97 to 99%) suggests that the Sensititre YeastOne colorimetric method may be a valuable tool for determining the susceptibility of filamentous fungi to voriconazole.

In recent years, opportunist filamentous fungi have come to pose a serious threat to immunosuppressed patients. The incidence of *Aspergillus* infection has risen over the last 20 years to become the most frequent life-threatening invasive fungal infection (9, 27). Increased incidence appears to be linked to the growing use of immunosuppressive drugs for the treatment of autoimmune diseases, to the spread of AIDS, to the increasing use of chemotherapy against various tumors, and to the large number of transplants now performed (8, 25, 27).

Many emerging fungi pose problems of intrinsic resistance to classical antifungal agents; this is exacerbated by simultaneous acquired in vitro resistance, i.e., resistant clinical isolates belonging to previously susceptible species (37, 39). Increased resistance has led to the need for new antifungal agents.

These new agents, including voriconazole (5), offer enhanced clinical efficacy coupled with lower toxicity. In 2002, the National Committee for Clinical Laboratory Standards (NCCLS) issued a protocol for testing the susceptibility of filamentous fungi, published as document M38-A (34). This standardization has enabled valid comparison of the results obtained in different susceptibility tests.

However, these standardized methods are complex and time-consuming, and the growing demand for tests of this sort has prompted a need for more accessible commercial methods. One such method is the Sensititre YeastOne colorimetric antifungal panel (13).

The present study compared two microdilution methods for testing the susceptibility of various species of filamentous fungi to voriconazole, the reference method (NCCLS M38-A) and Sensititre YeastOne, and compared results with those reported by other authors with the reference method.

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MATERIALS AND METHODS

Recovery and identification of strains. A total of 244 strains were isolated from various clinical specimens as follows: 102 bronchoaspirates, 98 sputum samples, 14 ear exudate samples, 10 bronchoalveolar lavage samples, 6 biopsy samples, 3 nasal exudate samples, 3 cerebrospinal fluid samples, 2 pharyngeal exudate samples, 2 blood culture samples, 1 brain abscess sample, 1 granuloma sample, 1 conjunctival exudate sample, and 1 pulmonary valve supernatant sample.

The following species were isolated: 223 *Aspergillus* (136 *Aspergillus fumigatus*, 37 *A. niger*, 26 *A. terreus*, 16 *A. flavus*, 7 *A. nidulans*, and 1 *A. ustus*), 14 *Fusarium* (8 *Fusarium moniliforme* and 6 *F. oxysporum*), 6 *Scedosporium apiospermum*, and 1 *Rhizomucor pusillus* strain.

Strains were identified by simple microbiological methods, including gross inspection, and by microscopic examination after microculture and growth on different media (22).

Fungal isolates. All isolates were stored as suspensions in sterile distilled water at −70°C until the study was performed. Prior to testing, each isolate was subcultured on potato dextrose agar (34).

Antifungal agents. (i) NCCLS method:. Standard voriconazole antifungal powder was supplied by Pfizer, Inc., Central Research Division (Groton, Conn.). Dilutions were prepared according to NCCLS guidelines. Final voriconazole concentrations ranged from 0.03 to 16 μg/ml.

(ii) Sensititre YeastOne method. Disposable 96-well plates with incorporated Alamar Blue contained twofold serial dilutions of dried antifungal agents: amphotericin B (0.008 to 16 μg/ml), fluconazole (0.125 to 256 μg/ml), itraconazole (0.008 to 16 μg/ml), ketoconazole (0.008 to 16 μg/ml), flucytosine (0.03 to 64 μg/ml), and voriconazole (0.008 to 16 μg/ml.). Voriconazole occupied the seventh of the eight rows in each plate. The first well on the first row was used as a control (1A) (11, 29).

(iii) Culture media. RPMI 1640 was used both for the reference method M38-A and, with 1.5% added glucose, for the Sensititre YeastOne method (29).

Inoculum preparation. (i) NCCLS method. The inoculum was prepared according to M38-A guidelines (34).

(ii) Sensititre YeastOne method. Isolates were subcultured on potato dextrose agar and incubated at 30°C for 7 to 14 days. After colony growth, a sterile bacteriological loop was scraped gently over the surface growth to dislodge aerial mycelia (conidia and hyphal fragments);a loopful of culture was then suspended in sterile distilled water containing 1% Tween 80 (4). The optical density was

TABLE 1. Susceptibility of filamentous fungal species to voriconazole tested using Sensititre YeastOne

Species (no. of strains)	MIC range (μg/ml)		MIC_{50} (μg/ml)		MIC_{90} (μg/ml)	
	Sensititre	NCCLS[a]	Sensititre	NCCLS[a]	Sensititre	NCCLS[a]
A. fumigatus (136)	0.016–1	0.016–1	0.25	0.125	1	0.5
A. niger (37)	0.125–1	0.125–1	0.25	0.25	0.5	0.5
A. terreus (26)	0.06–0.5	0.03–0.25	0.125	0.125	0.5	0.5
A. flavus (16)	0.06–0.5	0.03–1	0.25	0.25	0.5	0.5
A. nidulans (7)	0.125–0.25	0.125–0.25	0.125	0.125	0.25	0.25
A. ustus (1)	0.06	0.125				
F. moniliforme (8)	0.5–4	0.5–2	0.25	0.25	0.5	0.5
F. oxysporum (6)	0.5	0.25–0.5	0.25	0.25	0.5	0.5
S. apiospermum (6)	1	0.5–1	0.5	0.5	0.5	1
R. pusillus (1)	2	2				

[a] Data are from NCCLS document M38-A.

adjusted to give a final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml by McFarland standard.

Growth medium containing the inoculum suspension was added to plates containing antifungal agents at various concentrations, enabling dissolution of the final antifungal concentration and the pH indicator. Plates were sealed and incubated at 35°C for 48 to 72 h depending on the species.

Reading and interpretation of results: (i) NCCLS method. Readings were performed with the aid of an inverted mirror. As is customary for azole compounds such as voriconazole, the MIC was defined as the lowest concentration that produced a 100% reduction in fungal growth compared to that of the drug-free growth control (10, 16, 28).

Sensititre YeastOne method. Plates were incubated until a change in color from blue (indicative of no growth) to red (indicative of growth) was observed in the growth control well. MICs were determined after 48 to 72 h of incubation. Growth was evident as a change in the Alamar Blue growth indicator from blue to pink; this change facilitates clearer identification of cutoff points than the turbidimetric method, thus reducing the trailing effect characteristic of azole antifungal agents that hinders the interpretation of results when dilution techniques are used (13).

For other antifungal agents, the MIC is defined as the lowest drug concentration at which there is no change of color, i.e., the first blue well. For azoles, however, the change from pink to blue is not always complete, and there may be an intermediate purple color (due to the trailing effect) indicative of partial growth inhibition; in such cases, the MIC was defined as the lowest drug concentration resulting in a purple color (14).

The turbidity of wells was not taken into account when reading MICs. These data were used to calculate the MIC_{50} and the MIC_{90} (the antifungal MICs required to inhibit 50 and 90% of isolates, respectively) for genera and species of which more than five strains were tested.

(i) Quality controls. Each test included a reference strain in order to detect any alteration in the antifungal agent should the MIC obtained fail to fall within the reference range.

Four fungal strains with known susceptibility to voriconazole were used as quality controls: *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Aspergillus fumigatus* ATCC 204305, and *A. flavus* ATCC 204304 (1, 34).

RESULTS

The most frequently encountered genus was *Aspergillus*, which accounted for 223 of the 244 strains tested, i.e., 91.39% of total strains. The species most often identified was *A. fumigatus*, with 136 strains (55.73% of all strains). The highest percentage of clinical samples was from bronchoaspirates, which accounted for 102 of the 244 (41.80%) strains tested.

Using both methods, voriconazole MICs for the reference strains tested, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *A. fumigatus* 204305, and *A. flavus* ATCC 204304 were within the range specified in NCCLS documents M27-A2 and M38-A del NCCLS.

All strains produced detectable growth (pink color in the growth control well) after 48 and the longest time to grow was 72 h of incubation. The strains taking the longest time to grow were *A. fumigatus* (five strains), *A. niger* (two strains), most *Fusarium* strains (eight strains), and *S. apiospermum* (five strains).

Susceptibility to voriconazole, as tested by the Sensititre YeastOne method and the reference method, is shown in Table 1. The maximum MIC range for the various genera was ≤1 μg/ml, with the exception of a single strain of *F. moniliforme*, which recorded MICs of 4 μg/ml using Sensititre YeastOne and of 2 μg/ml using the NCCLS reference method. An MIC of 2 μg/ml was found with both methods for the only *R. pusillus* strain tested and for *F. moniliforme* with an MIC of 2 μg/ml by one method and an MIC of 4 μg/ml by the other, suggesting that they were both the least susceptible to voriconazole. TheMIC_{50} and MIC_{90} values obtained by using the two microdilution methods for the remaining genera also differed by ≤1 μg/ml.

Differences in MICs of no more than two dilutions between the two methods were used to calculate the percent agreement. Where differences exceeded two dilutions, there was considered to be no agreement (24).

For 86 of 136 (63%) *A. fumigatus* isolates the voriconazole MICs were determined to be the same by both methods, for 32 of 136 (24%) isolates the voriconazole MICs differed by one dilution, and for 16 of 136 (12%) isolates the voriconazole MICs differed by two dilutions. For 28 of 37 (76%) *A. niger* isolates the voriconazole MIC was determined to be the same by both methods, 7 of 37 (19%) isolates differed in voriconazole MIC by one dilution, and 1 of 37 (3%) isolates differed in voriconazole MIC by two dilutions. For 21 of 26 (81%) *A. terreus* isolates the voriconazole MIC was the same as determined by both methods, 3 of 26 (12%) isolates differed in voriconazole MIC by one dilution, and 1 of 26 (4%) isolates differed in voriconazole MIC by two dilutions. For 14 of 16 (88%) *A. flavus* isolates the voriconazole MIC was the same as determined by both methods, 1 of 16 (6%) isolates differed in voriconazole MIC by one dilution, and 1 of 16 (6%) isolates differed in voriconazole MIC by two dilutions.

The highest rate of agreement between the two methods (99%) was found for *A. fumigatus*, *A. nidulans*, *A. ustus*, and *R. pusillus*, although the number of strains tested for the latter two species was not significant. Strain-by-strain comparison of

TABLE 2. Number of dilutions by which MICs differed as determined by two microdilution methods

Species (no. of strains)	No. of dilutions by which MICs differed				
	0	1	2	>2	% Agreement
A. fumigatus (136)	86	32	16	2	99
A. niger (37)	28	7	1	1	98
A. terreus (26)	21	3	1	1	97
A. flavus (16)	14	1	1		98
A. nidulans (7)	6	1			99
A. ustus (1)		1			99
F. moniliforme (8)	5	2	1		97
F. oxysporum (6)	4	2			98
S. apiospermum (6)	2	3	1		98
Rhizomucor pusillus (1)					99

the results obtained by the two methods disclosed 97 to 99% agreement for all species tested.

Breakdown of these results by species enabled examination of the differences between the two methods: for *A. fumigatus* (n = 136) the same voriconazole MIC was recorded for 86 strains, a difference of one dilution was found for 32 strains, and of two dilutions for 16 strains; for 2 additional strains, there was no agreement between microdilution methods. For *A. niger*, the same voriconazole MIC was recorded for 28 of the 37 strains tested, a difference of one dilution was found for 7 strains and of two dilutions for 1 strain; there was no agreement (>2-dilution difference) for only one strain. The full results for all strains are shown in Table 2.

DISCUSSION

These results were compared to the susceptibility findings of other authors with voriconazole. The voriconazole MIC_{90} obtained here for *A. flavus* and *A. niger* agreed with that 0.5 μg/ml reported by Murphy et al. (33) and differed by 1 or 2 dilutions from the 1 μg/ml obtained by Espinel-Ingroff (15) and from the values recorded by Oakley (35), who report a MIC_{90} of 1 μg/ml for *A. niger* and of 2 μg/ml for *A. flavus*. The MIC_{90} values obtained here also matched those reported for *A. flavus* by Johnson et al. (20).

Our results for *A. fumigatus* agreed with those of both Espinel-Ingroff (15) and Oakley (35) but differed by one or two dilutions from those reported by Johnson (20) and Murphy (33), respectively. The highest MIC obtained by Dannaoui et al. (7) for *A. fumigatus* was 0.5 μg/ml, i.e., a difference of one dilution with respect to the present results.

Emerging invasive *Scedosporium* and *Fusarium* opportunistic infections are thought to be among the most aggressive and treatment-refractory infections (18). Since they are often resistant to both amphotericin B and the azole group, they are associated with high mortality rates (2); hence, the need for a drug with activity against both fungi.

In the present study, *S. apiospermum* displayed in vitro susceptibility to voriconazole (MIC = 0.5 to 1 μg/ml) (6, 12, 23, 39). Other authors report similar or even greater activity. Johnson et al. (20) found a voriconazole MIC_{90} of 0.5 μg/ml, with an MIC range of 0.06 to 0.25 μg/ml for the 10 strains tested; Espinel-Ingroff (15) reported a range of 0.06 to 1 μg/ml for 15 strains tested, with a voriconazole MIC_{90} similar to that recorded by Johnson et al. (20).

As is the case with yeast-like organisms, there are reports of potential cross-resistance between voriconazole and other azoles. However, no significant resistance was observed here. Other authors (32, 38) also report that an increase in the MIC for itraconazole tends to be matched by an increase in that of voriconazole.

The 10 *Fusarium* strains tested displayed greater susceptibility to voriconazole (MICs of 0.25 to 4 μg/ml as determined by Sensititre and the reference method, respectively) than that reported by other authors, including Johnson et al. (20) and Espinel-Ingroff (15), who obtained MICs of between 2 and 8 μg/ml.

The results obtained here confirm the in vitro efficacy of voriconazole as an alternative treatment for invasive infection by filamentous fungi, particularly by *Aspergillus* species resistant to other antifungal agents (32).

Voriconazole is currently an excellent option for the treatment of fungal infections; it offers perhaps the broadest spectrum of activity, greater even than that of amphotericin B, with the additional benefit of oral and parenteral administration (21, 36).

The clinical value of the MIC as a predictor of the resistance or susceptibility of filamentous fungi to voriconazole has yet to be established in clinical studies. However, results from animal models and clinical trials suggest that voriconazole is effective in treating *Aspergillus* and *Scedosporium* infections (9, 17, 33); some trials report a good correlation between voriconazole MICs and clinical results (17).

Herbrecht et al. (19) found a better overall response to voriconazole than to amphotericin B in immunocompromised patients with invasive aspergillosis infection. Voriconazole was better tolerated than amphotericin B, with fewer adverse effects and a lower rate of abandonment. Survival rates were also higher with voriconazole.

No studies have yet compared susceptibility of filamentous fungi as measured by Sensititre YeastOne versus the reference method. However, comparisons of both methods with other antifungal agents such as amphotericin B and itraconazole have yielded agreement rates of 77 to 93.4% for itraconazole and 66 to 90.2% for amphotericin B (30, 31).

Yamaguchi et al. (40) studied the susceptibility of *Aspergillus* species to a range of antifungal agents, including voriconazole, by using the reference method and a colorimetric method, obtaining 94% agreement.

The efficacy of voriconazole against the species tested, as measured by using Sensititre YeastOne, was very similar to that reported by other authors (3, 15, 20, 24, 26, 33) by using the NCCLS reference method M38-A, thus underlining the value of the Sensititre method for determining the susceptibility of filamentous fungi to voriconazole.

In summary, the high agreement rate (97 to 99%) between the two methods for all species tested confirms that Sensititre YeastOne is an efficient and effective alternative to the reference method for determining the susceptibility of filamentous fungi to voriconazole in clinical microbiology laboratories.

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